Volume 157, number 2 FEBS 0540 July 1983

Stimulation by thyrotropin and cyclic AMP of the proliferation of quiescent canine thyroid cells cultured in a defined medium containing insulin

Pierre P. Roger, Patricia Servais and Jacques E. Dumont

Institute of Interdisciplinary Research (IRIBHN), School of Medicine, Free University of Brussels, Campus Erasme, Route de Lennik 808, B-1070 Brussels and *Biology Department, Euratom, Brussels, Belgium*

Received 6 May 1983

We have developed serum-free primary cultures of differentiated follicular dog thyroid cells which allow the study of the hormonal control of cell proliferation. The cooperation of insulin and increasing cellular cyclic AMP by thyrotropin triggers the DNA synthesis and the proliferation. Dog thyroid cells are an example of a system in which cyclic AMP is a sufficient signal to stimulate the proliferation in quiescent cells.

Thyroid *Thyrotropin* *Cyclic AMP* *Forskolin* *DNA synthesis* *Proliferation* *Serum-free culture*

1. INTRODUCTION

The possibility that cyclic AMP (cAMP) may regulate normal cell proliferation is the subject of a plethoric but controversial literature. From the first studies using mainly cultured fibroblasts, cAMP has been proposed as a universal intracellular negative signal for proliferation [1]. An objection to many of these studies has been that these effects were elicited by high concentrations of analogues of cAMP and could be regarded as non-specific [2,3]. Now cAMP has been shown to have a positive influence on the proliferation of a growing number of cultured cells from various origins, including hepatocytes [4–6] and 3T3 fibroblasts [7], the systems in which the understanding of proliferation is the most advanced. The problem has a special interest in endocrine epithelial tissues in which activity and growth in vivo are specifically controlled by pituitary trophic hormones (ACTH, FSH, LH, TSH) whose main action is to stimulate adenylate cyclase. However, in general, these trophic hormones have rather inhibitory effects on the proliferation of their target cells in culture [8–13], leading to the speculation that they may not be direct mitogens in vivo [8,12,13].

Little is known about hormonal effects on thyroid cell proliferation. In a few culture systems, thyrotrophin (TSH) stimulates DNA synthesis and proliferation [14–16], but not in several others [13,17,18]. In terms of mechanisms, dog thyroid cells in primary culture in monolayer remain a unique model as, in this sytem, the growth-promoting action of TSH has been shown to be reproduced by increasing intracellular cAMP levels [16]. However, the interpretaion of our results and the characterization of the TSH effect have been limited by the fact that the serum-containing medium by itself promotes cell multiplication which is only enhanced by thyrotropin. Serum is a complex, variable, not fully characterized hormonal mixture which may mask or affect the response of cells to purified hormones or factors [19]. Therefore, we describe here serum-free, defined culture conditions in which the differentiated naturally quiescent dog thyroid cells can be induced to synthesize DNA and to proliferate by

Published by Elsevier Science Publishers B.V.
00145793/83/$ 3.00

TSH acting via cAMP. The only hormone required to obtain the TSH effect on proliferation, is insulin.

2. MATERIALS AND METHODS

2.1. *Materials*

Collagenase (150 U/mg) was purchased from Worthington Chemical Co. (Freehold NJ). Dulbecco's modification of minimum essential medium (DMEM), Ham's F12 medium, MCDB 104 medium, glutamine, penicillin-streptomycin and amphotericin B (fungizone) were obtained from Flow Labs (Irvine). Bovine insulin and transferrin were purchased from Collaborative Research (Waltham MA). Glycyl-histidyl-lysyl acetate and somatostatin were Sigma products (St Louis MO). Bovine TSH (1 U/mg) was from Armour Pharmaceutical Co. (Chicago IL) or, when purified (40 U/mg), was a generous gift of Dr Pierce (UCLA CA). Cholera toxin was provided by Schwarzmann (Division of Decton-Dickinson, Orangeburg NY). Forskolin was from Hoechst Pharmaceuticals (Bombay), dibutyryl cyclic AMP (dbcAMP) from Boehringer Pharmaceutical (Mannheim) and RO 20-1724 was a gift of Hoffman-La Roche (Nutley NJ). [*methyl*-^{3}H]Thymidine (40 Ci/mmol) was from the Radiochemical Centre (Amersham).

2.2. *Cell culture*

The dog thyroid cells were cultured as in [16], but in a serum-free defined medium. Briefly, the thyroid tissue was digested by collagenase so that the resulting suspension consisted mainly of fragmented and intact follicles. These follicles were seeded in 35 mm tissue culture-treated plastic Petri dishes, and, in 1 day, adhered to the substratum while a monolayer developed. The seeding was realized so that 5×10^4–2×10^5 cells attached to the dish after 1 day and 1 medium change. The cells were cultured in the following mixture which constitutes the control medium: DMEM + F12 + MCDB 104 (2:1:1, by vol.) with 2 mM glutamine, supplemented by 10 μg insulin/ml, 1.25 μg transferrin/ml, 10 ng glycyl–histidyl–lysyl–acetate/ml, 10 ng somatostatin/ml and 40 μg ascorbic acid/ml. Antibiotics, penicillin 100 U/ml, streptomycin 100 μg/ml and amphotericin B 2.5 μg/ml were also added. The Petri dishes were maintained in a water-saturated incubator at 37°C in an atmosphere of 5% CO_2 in air. The medium was renewed and TSH or other effectors were added as indicated.

2.3. *Proliferation assays*

Cell multiplication curves were obtained from cell DNA measurements. Our cell DNA assay described in [16], uses the increase of fluorescence of ethidium bromide when complexed with nucleic acids. It has been verified that an increase in DNA quantity per dish reflected an increase in cell number [16].

DNA synthesis was estimated by the incorporation of [^{3}H]thymidine into 10% trichloroacetic acid-precipitable material or by the frequency of the [^{3}H]thymidine-labelled nuclei as estimated by autoradiography. The cells in the Petri dishes were incubated for 24 h time-periods or for determined times after the addition of a proliferation stimulator, in the complete medium in which thymidine was increased to 3×10^{-5} M, supplemented with 10^{-4} M deoxycytidine and 10 μCi [^{3}H]thymidine/ml. The cells were stopped by 10% trichloroacetic acid or, for autoradiography, fixed by methanol, and then extensively washed. Autoradiographies were performed as in [20] directly in the Petri dishes. The cells were stained with toluidine blue (1%) and the proportion of labelled nuclei was evaluated by counting at least 500 nuclei from different microscopical fields. Silver grains were exclusively restricted to the nuclei and, in some cases, it was observable that nucleoli remained unlabelled.

Results were expressed as means ± ranges of measurements on duplicate Petri dishes. All results presented were confirmed in at least 3 independent cultures.

3. RESULTS

Plated directly on plastic tissue culture-treated Petri dishes in the serum-free defined medium, most intact or fragmented follicles very rapidly attached (about 50% in 15 min) and subsequently spread and developed a cell monolayer. Addition of TSH (10 μU–10 mU/ml) at any time after the formation of the monolayer, induced rapidly a conspicuous cytoplasmic arborization in the whole

cell population. This acute morphological effect of TSH is characteristic of the TSH-responsive epithelial thyroid cells in monolayer [13,21,22]; it was reproduced by treatments we used to increase intracellular cAMP levels; i.e., by cholera toxin (20 ng/ml), forskolin (10^{-5} M) [23,24] or by dbcAMP (5×10^{-5} M) in the presence of the inhibitor of phosphodiesterases RO 20-1724 (4×10^{-5} M). TSH and treatments increasing cAMP chronically stimulated our cells to actively concentrate radioiodide (a thyroid-specific marker of differentiation) at very high levels (cell to medium ratios were currently above 100) (not shown). This suggests that the cultures used in this study were purely constituted of follicular thyroid cells which all responded to TSH in a cAMP-dependent fashion.

3.1. *Stimulation of proliferation by TSH*

As seen in fig.1, in the absence of TSH, the cells were surviving and showed a little increase in DNA content per dish in the first days of culture. Addition of TSH (1 mU/ml), 1 day after seeding and its continuous presence in the culture medium resulted in a limited but significant proliferation.

The study of DNA synthesis by incorporation of 24 h pulses of [^{3}H]thymidine into 10% trichloroacetic acid-precipitable material is shown in fig.2. In control medium, the cells weakly incorporated

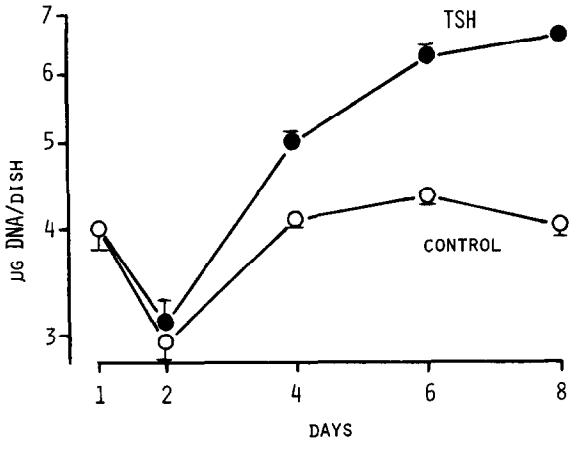

Fig.1. Stimulation of proliferation by TSH in defined conditions. The cells were seeded as in section 2. TSH (1 mU/ml) was added to control medium at day 1 and its presence was maintained throughout the culture period, while medium was renewed every other day.

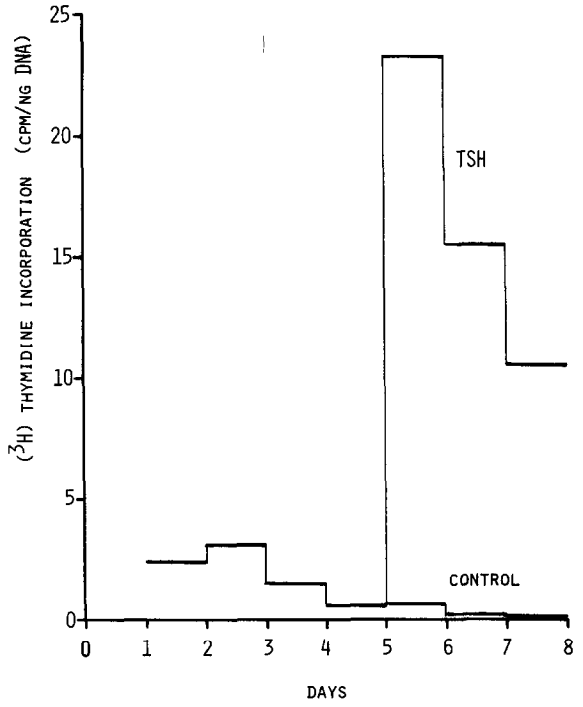

Fig.2. DNA synthesis by thyroid cells in response to TSH. The cells were seeded and cultured in the control medium as in section 2. At day 4, TSH (1 mU/ml) was added to some dishes and its presence was maintained for the rest of the culture period. The medium was renewed every day. [^{3}H]Thymidine (10 μCi/ml, 3×10^{-5} M) incorporation in the presence of deoxycytidine (10^{-4} M) into acid-insoluble material was determined for 24 h periods and normalized to the quantity of cell DNA/dish.

thymidine in the first days following initiation of cultures. After day 4, virtually no incorporation was observed, the cells remaining quiescent. There was no concomitant decrease of the DNA content which suggests that no significant cell death occurred in our serum-free cultures, at least until day 8. Addition of TSH (1 mU/ml) at day 4 (i.e., to stationary cells) triggered, 24 h thereafter, a burst of thymidine incorporation (about 50 × increase) which decreased during the following days.

Autoradiography of [^{3}H]thymidine labelled nuclei 24 h after addition of TSH (1 mU/ml) (fig.3). There was therefore a 24 h time lag after TSH addition. 60 h after TSH addition, a majority of cells were labelled. Labelling was randomly distributed in the follicle-derived colonies, but the colonies were not all equally labelled. Mitoses and sub-

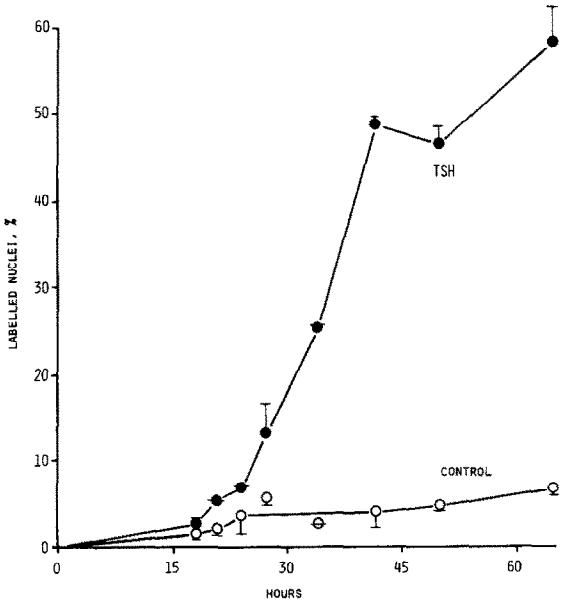

Fig.3. Accumulation of [^{3}H]thymidine-labelled nuclei after addition of TSH. The cells were cultured for 4 days in control medium with one medium change at day 2. TSH (1 mU/ml) was added at day 4 (0 h) and its presence maintained for the rest of the experiment while the medium was still changed at day 5. [^{3}H]Thymidine was present from 0 h until the times indicated. At the indicated times, the cells were fixed in methanol, rinsed and processed for autoradiography. The fraction of labelled nuclei was determined.

Table 1

Effect of TSH and treatments increasing cAMP on [^{3}H]thymidine incorporation in the DNA of dog thyroid cells

	cpm/ng DNA	% labelled nuclei
Expt I		
Control	1.28 ± 0.29	1.9 ± 0.5
TSH (1 mU/ml)	24.0 ± 0.2	53.2 ± 4.5
Pure TSH (25 ng/ml)	18.6 ± 1.2	59.8 ± 4.3
dbcAMP (5.10^{-5}) + Ro 20-1724 (4.10^{-5} M)	n.d.	44.3 ± 8.1
Cholera toxin (10 ng/ml)	28.9 ± 1.9	52.6 ± 5.9
Expt II		
Control	1.78 ± 0.21	n.d.
TSH (1 mU/ml)	19.7 ± 2.3	n.d.
Forskolin (10^{-5} M)	19.1 ± 2.5	n.d.

n.d. = not determined

The experiments were performed as in fig.2 and 3. TSH or the other effectors were added at day 4. [^{3}H]Thymidine incorporation was for 24 h between days 5 and 6. DNA synthesis was estimated by the incorporation of radioactivity into acid-insoluble material, normalized to cell DNA, or by the fraction of labelled nuclei revealed by autoradiography

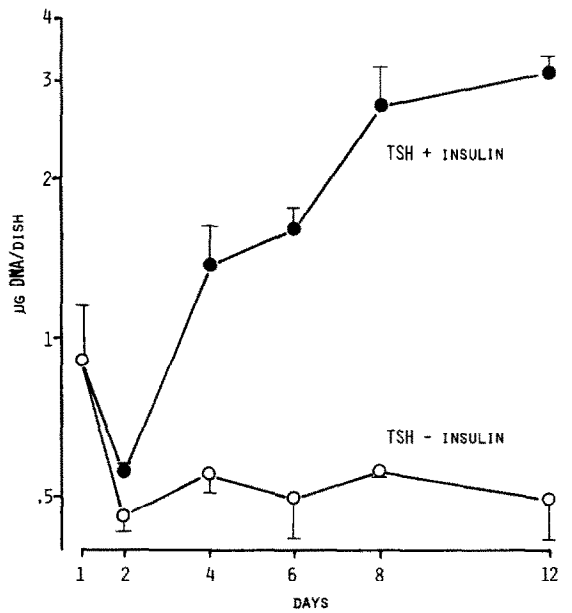

Fig.4. Effect of TSH on the proliferation of cells cultured in presence or in absence of insulin. The cells were seeded in the control medium where insulin was omitted. At day 1, TSH (1 mU/ml) was added in presence or in absence of insulin (10 µg/ml). The presence of both hormones was maintained for the rest of the culture period while the medium was renewed every other day.

sequent divisions of labelled cells were observed. Concentration–action curves of TSH added at day 4 on the thymidine incorporation and on DNA accumulation were similar. The effects were observed at 50 µU/ml, half-maximal at 100 µU/ml and maximal at 1 mU/ml. The maximal effects were similar whether crude bovine TSH preparation (~1 U/mg) or pure bovine TSH (40 U/mg) was used (table 1).

Table 2

Effect of TSH on [^{3}H]thymidine incorporation into DNA in the presence or in the absence of insulin

	% labelled nuclei
Insulin (10 μg/ml)	2.83 ± 1.0
TSH (1 mU/ml)	3.31 ± 1.06
TSH + insulin	33.5 ± 4.7

The cells were seeded and cultured for 2 days in control medium lacking insulin. At day 2, insulin (10 μg/ml) was added to some dishes, and its presence maintained for the rest of the experiment. At day 4, TSH (1 mU/ml) was added to cells cultured with or without insulin, and its presence maintained for the rest of the culture. DNA synthesis was evaluated by incorporation of [^{3}H]thymidine for 24 h between day 5 and day 6, into nuclei and revealed by autoradiography

3.2. *Stimulation of proliferation by cAMP*

TSH acutely and chronically increases cellular cAMP levels in dog thyroid cells cultured in monolayer [16,25]. As seen in table 1, the maximal TSH effect on the initiation of DNA synthesis was quantitatively reproduced by treatments which activate adenylate cyclase and increase cellular cAMP levels; i.e., by the universal specific activators of adenylate cyclase, cholera toxin [26] and the diterpene forskolin [27]. The effect of TSH was also reproduced by dbcAMP (5×10^{-5} M) in the presence of Ro 20-1724 (4×10^{-5} M), an inhibitor of cyclic nucleotide phosphodiesterases.

3.3. *Importance of insulin in the stimulation of proliferation*

In our defined control medium, the only hormone which has a recognized role in the control of cell proliferation, is insulin [28]. Fig.4 shows that, in thyroid cells surviving in absence of insulin, TSH failed to increase the DNA quantity per dish, and did not trigger a significant DNA synthesis (table 2).

4. DISCUSSION

The present study describes for the first time completely defined serum-free culture conditions which allow the investigation of the hormonal control of proliferation of normal differentiated thyroid cells in primary culture. The defined medium supports cell adherence and spreading, differentiation evaluated by the capacity of cells to trap iodide and to respond to TSH, and the stimulation of multiplication in quiescent cells. We use relatively short term primary cultures of cells which have not, in their majority, proliferated in vitro before hormone addition. These cells may not be suspected to be genetically transformed or selected by their in vitro culture conditions, as in the case of cloned cell lines. Fibroblast-like cells were never seen in our cultures. Possibly the few fibroblasts seeded with follicles could not attach to a plastic substratum in absence of serum.

Under these conditions, we demonstrate that TSH triggers the synthesis of DNA and proliferation in quiescent follicular canine thyroid cells. This effect is direct, requires neither the presence of other thyroid cell types, nor unidentified serum factors, nor the action of the purified hormone-like growth factors (epidermal and fibroblast growth factors). Such growth factors also stimulate the proliferation of canine thyroid cells [29] (in preparation). The TSH effect was obtained in the range of plasma TSH concentrations which cause the thyroid hyperplasia in vivo.

Insulin is the only hormone which must be present in the culture medium to obtain the effect of TSH on proliferation. The two hormones work in synergy, cooperate to finally induce DNA synthesis and proliferation. Insulin is a general mitogenic factor for cultured cells. Its action may represent an anabolic permissive effect. However, at high concentrations, insulin acts as a weak analogue of the more specific mitogens the somatomedins [28]. We are currently investigating the respective roles of insulin and TSH in their synergistic stimulation of thyroid cell proliferation. We have preliminary indications that insulin exerts part of its effects at low concentrations, suggesting an action via its high affinity receptors (unpublished).

The main primary action of TSH on canine thyroid tissue [30] or on thyroid cells in culture [16,25] is to activate adenylate cyclase and to increase cellular cAMP levels. TSH exerts most of its acute and delayed functional effects on dog thyroid via cAMP [31]. The fact that universal specific activators of adenylate cyclase (cholera toxin and forskolin) and a rather low concentra-

tion of the hydrophobic analogue of cAMP, dbcAMP, in presence of an inhibitor of phosphodiesterases, completely reproduced the mitogenic effect of TSH, demonstrates that TSH exerts also its effect on proliferation via cAMP as intracellular secondary signal. TSH also exerts cAMP-independent effects, including an increase in phosphatidylinositol turnover and in Ca^{2+} translocation [30]. The final significance of these effects remains unknown. However, as most growth factors have such effects and as Ca^{2+} is considered to have a key role in the control of proliferation [4,32], it was possible that such mechanisms may have a role in the control of thyroid cell proliferation by TSH. Our findings that the TSH effect on proliferation may be completely reproduced by agents which specifically increase cAMP levels, does not support this hypothesis, at least in the case of the dog thyroid. A rat thyroid cell line (FRTL) requires TSH and insulin in order to proliferate in a low-serum-hormone-supplemented medium [15], similarly to observations presented here. IN FRTL5, a cloned cell line derived from FRTL and adapted to grow in presence of 5% serum, dbcAMP does not reproduce the mitogenic effect of TSH [33] leading to the conclusion that TSH stimulates thyroid cell proliferation acting at least in part by cAMP-independent mechanisms. Obviously, such negative evidence needs to be confirmed with more potent and specific stimulations of the cyclic AMP system. This apparent discrepancy may be due to species differences, but the possibility that cells, as FRTL5, continuously propagated and cloned in presence of TSH, became dependent of cAMP-independent TSH effects not originally involved in the growth control, may certainly not be ruled out.

Many reports now suggest a positive influence of cAMP on the proliferation of cultured cells from a variety of tissues and species [4–7,12,34–42]. In general, in these systems, cholera toxin or cAMP derivatives increase the proliferation stimulated by serum or growth factors [4–6,12,34–39,42]. Like Swiss 3T3 fibroblasts [7], the dog thyroid cells cultured in defined conditions constitute a rare example of a model system in which an increase in cellular cAMP is sufficient to trigger the proliferation of a significant part of a quiescent cell population. This system is therefore a very useful model for the biochemical investigation of the molecular events involved in the control of growth by cAMP.

ACKNOWLEDGEMENTS

We are grateful to Dr John G. Pierce (UCLA, CA) for the gift of pure TSH and to Mrs D. Leemans for typing the manuscript. This work was done within the framework of Euratom contract B10-C-360-81-B, and partially thanks to grant 3.4536.81 of the Fonds de la Recherche Medicale. P.P.R. is a research fellow of the Institut pour l'Encouragement de la Recherche Scientifique dans l'Industrie et l'Agriculture (IRSIA).

REFERENCES

[1] Pastan, I.H., Johnson, G.S. and Anderson, W.B. (1975) Annu. Rev. Biochem. 44, 291–522.
[2] Friedman, D.L. (1976) Physiol. Rev. 56, 652–708.
[3] Martin, T.F.J. and Kowalchyk, J.A. (1981) Science 213, 1120–1122.
[4] Whitfield, J.F., McManus, J.P., Rixon, R.H., Boynton, A.L., Youdale, T. and Swierenga, S. (1976) In Vitro 12, 1–18.
[5] Koch, K.S. and Leffert, H.L. (1980) Ann. NY Acad. Sci. 349, 111–127.
[6] McGowan, J.A., Strain, A.J. and Bucher, N.L. (1981) J. Cell. Physiol. 108, 353–363.
[7] Rozengurt, E. (1982) J. Cell. Physiol. 112, 243–250.
[8] Gospadorowicz, D. and Gospadorowicz, F. (1974) Endocrinology 96, 458–467.
[9] Ramachandran, J. and Suyama, A.T. (1975) Proc. Natl. Acad. Sci. USA 72, 113–117.
[10] Hornsby, P.J. and Gill, G.N. (1977) J. Clin. Invest. 60, 342–352.
[11] Simonian, M.H., White, M.L. and Gill, G.N. (1982) Endocrinology 111, 919–927.
[12] Yang, J., Richards, J., Guzman, R., Imagawa, W. and Nandi, S. (1980) Proc. Natl. Acad. Sci. USA 77, 2088–2092.
[13] Westermark, B., Harlsson, F.A. and Walinder, O. (1979) Proc. Natl. Acad. Sci. USA 76, 2022–2026.
[14] Nitsch, L. and Wollman, S.H. (1980) Proc. Natl. Acad. Sci. USA 77, 2743–2747.
[15] Ambesi-Impiombato, F.S., Parks, L.A.M. and Coon, H.G. (1980) Proc. Natl. Acad. Sci. USA 77, 3455–3459.
[16] Roger, P.P., Hotimsky, A., Moreau, C. and Dumont, J.E. (1982) Mol. Cell. Endocrinol. 26, 165–176.
[17] Fayet, G. and Hovsépian, S. (1979) Biochimie 61, 923–930.
[18] O'Connor, M.K., Malone, J.F. and Cullen, M.J. (1980) Acta Endocrinol. Suppl. 231.

[19] Barnes, D. and Sato, G. (1980) Anal. Biochem. 102, 255–270.
[20] Gershenson, L.E., Berliner, J. and Young, J. (1974) Cancer Res. 34, 2873–2880.
[21] Rapoport, B. and Jones, A.L. (1978) Endocrinology 102, 175–180.
[22] Westermark, B. and Porter, K. (1982) J. Cell. Biol. 94, 42–50.
[23] Fradkin, J.E., Cook, H., Kilhoffer, M.-C. and Wolff, S. (1982) Endocrinology 111, 849–856.
[24] Van Sande, J., Cochaux, P., Mockel, J. and Dumont, J.E. (1983) Mol. Cell. Endocrinol. 29, 109–119.
[25] Rapoport, B. (1975) Endocrinology 98, 1189–1197.
[26] Gill, D.M. (1977) Adv. Cyclic Nucl. Res. 8, 85–89.
[27] Seamon, K.B. and Daly, J.W. (1981) J. Cyclic Nucl. Res. 7, 201–224.
[28] Strauss, D.S. (1981) Life Sci. 29, 2131–2139.
[29] Roger, P.P. and Dumont, J.E. (1982) FEBS Lett. 144, 209–212.
[30] Dumont, J.E., Takeuchi, A., Lamy, F., Gervy-Decoster, C., Cochaux, P., Roger, P., Van Sande, J., Lecocq, R. and Mockel, J. (1981) Adv. Cyclic Nucl. Res. 14, 479–489.
[31] Dumont, J.E. (1971) Vitamins Hormones 29, 287–412.
[32] Berridge, M.J. (1975) J. Cyclic Nucl. Res. 1, 305–320.
[33] Valente, W.A., Vitti, P., Kohn, L.D., Brandi, M.L., Rotella, C.M., Toccafondi, R., Tramontano, D., Aloj, S. and Ambesi-Impiombato, F.S. (1983) Endocrinology 112, 71–79.
[34] Marcello, C.L. (1979) Exp. Cell Res. 120, 201–210.
[35] Raff, M.C., Hornby-Smith, A. and Brockes, J.P. (1978) Nature 273, 672–673.
[36] Taylor-Papadimitriou, J., Purkis, P. and Fentiman, I.S. (1980) J. Cell. Physiol. 102, 317–321.
[37] Davison, P.M. and Karasek, M.A. (1981) J. Cell. Physiol. 106, 253–258.
[38] Eisinger, M. and Marko, O. (1982) Proc. Natl. Acad. Sci. USA 79, 2018–2022.
[39] Mayer, T.C. (1982) Dev. Biol. 94, 509–514.
[40] Rabinovitch, A., Blondel, B., Murray, T. and Mintz, D.H. (1980) J. Clin. Invest. 66, 1065–1071.
[41] Taub, M., Saier, M.H. jr, Chuman, L. and Hiller, S. (1983) J. Cell. Physiol. 114, 153–161.
[42] Tsang, B.K., Rixon, R.H. and Whitfield, J.F. (1980) J. Cell. Physiol. 102, 19–26.